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(54) Anti Cancer Therapy and Compositions

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ABSTRACT

This invention is of prospective interest to ZENECA but rights in it would have to be acquired from the inventors. The protection available is limited by the inventors prior publications

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ANTICANCER THERAPY AND COMPOSITIONS

DESCRIPTION

Technical field

The present invention relates to the use of a protein complex as an anticancer agent.

The object of the present invention is to obtain an anticancer composition for prophylaotic or therapeutic treatment of cancers.

In a paper, Apoptosis induced by a human milk protein Hakansson et al, *Proc Nat Acad Sci USA* 92 pp 8064-8068 it is disclosed that a component of human milk in a particular physical state, multimeric α-lactalbumin (MAL) killed a number of transformed embryonic and lymphoid cells whilst sparing mature epithelial elements. It concludes that further analysis of the mechanism by which MAL induces apoptosis in transformed epithelial cells could lead to the design of anti tumour agents.

We have now found that MAL is itself effective as an anti cancer agent when introduced directly into a mammalian body and that any adverse effects on lymphoid cells are acceptably low in *in vivo* tests. Surprisingly therefore MAL itself can be injected into the mammalian body or supplied in the form of a controlled release source which comprises MAL which can be implanted into the mammalian body as an anti cancer therapy.

In order to introduce this multimer into a mammalian body in this manner it is necessary that it be sterile.

The invention comprises a sterile injectable composition for use in the treatment of cancer in mammals which comprises multimeric α-lactalbumin in a pharmaceutically acceptable diluent. The preferable diluent is water. By "injectable" we mean injectable into any part of a mammalian, suitably human body. Injection into a vein, the peritoneum, muscle, under the skin or elsewhere may be appropriate according to the circumstances.

The pH of such a composition is suitably 6 to 8. Preferably it comprises 0.1 to 200 g/litre by weight of multimers of alpha-lactalbumin. It may also comprise monomeric alpha-lactalbumin.

The invention also comprises a method of processing mammalian body fluids outside the mammalian body which comprises adding a sufficient quantity of a multimer of α-lactalbumin to kill substantially all of any cancer

cells contained in the body fluid. Our data indicates that such fluids can be introduced into donors body or introduced into the body of another appropriate recipient.

The invention also comprises a composition which comprises a human body fluid outside the human body and a sufficient quantity of a multimer of α-lactalbumin to kill substantially all of any cancer cells contained in the body fluid.

In one form of the invention a multimer of alpha-lactalbumin is added to human blood for transfusion in a sufficient amount to kill any cancer cells. By this means the likelihood of inadvertent transmission of leukaemia from a donor to a recipient is reduced. A concentration of 0.01 to 2 g/litre and preferably 0.01 to 0.2 g per litre is added.

The injectable composition as aforesaid may be preserved in sterile condition by storage in sealed containers, for example preloaded syringes or intravenous infusion bags. These may each contain a single dosage amount of the multimer (MAL). For an adult patient this is suitably 0.15 to 15 g of the multimer, preferably administered at intervals of four to twenty four hours.

The multimer may be introduced into the mammalian body as a sterile composition which comprises a solid containing multimeric α-lactalbumin for insertion into a mammalian body which solid acts as a controlled release source of multimeric α-lactalbumin.

Sterile products may be produced by sterilising alpha-lactalbumin, either as such or when present in milk and converting it to the multimer, carrying out all steps subsequent to the initial sterilisation under sterile conditions or if desired the final product may be sterilised. As the multimers are sensitive to heat it is preferred that sterilization procedures carried out on material containing the multimers should not involve heating; it is preferred that sterilisation should be by radiation in this case.

A further object is to obtain a composition which is active on cancers on low differentiated cells such as epithelial cells as lung cells, bronchial cells, kidney, bladder, mammery and small-intestinal cells and non-epithelial cells such as fibroblast cells (connective tissue).

Background of the Invention

Eukaryotic cells are genetically programmed to die; a process referred to as programmed cell death (PCD). The term was coined in 1965 by Lockshin and Williams to describe the developmentally regulated death of specific larval muscles (1). The PCD concept is used in developmental biology to describe the loss of specific cells in response to a physiological stimulus; a normal event in the development of an organism. It is also used to denote cell death that requires de novo gene expression. Cells undergoing PCD change their morphology; the nuclear chromatin is condensed along the inner surface of the nuclear membrane, the organelles are packed and shed from the cell in membrane-surrounded vesicles (apoptotic bodies) marked with recognition molecules for the tissue macrophages. This morphologic appearance was first described in 1972 by Kerr, Wyllie and Currie, who named the phenomenon apoptosis after the Greek "falling of leaves" (2). Coincident with the morphological changes proteins and chromatin are digested by endogenous proteases and nucleases that cleave various target proteins and DNA. Chromatin cleavage with the formation of high- and low-molecular weight DNA fragments is the main biochemical hallmark of apoptosis (3, 4). The terms PCD and apoptosis are often intermixed although PCD signifies cell death resulting from activation of gene expression and apoptosis the changes in morphology that result from this process.

Apoptosis is induced by addition or withdrawal of physiologic or noxious stimuli. Deprivation of hormones leads to apoptosis in hormone-dependent tissues (5). Growth factors influence tissue growth and differentiation; in the absence of these stimuli cells undergo apoptosis. Apoptosis is also activated when cells are stimulated by noxious agents like irradiation, microbial toxins, or mediators like TNF produced in response to tissue injury or disease (6-9).

Unlike normal cells, tumour cells may fail to activate the apoptotic pathway. Indeed, a characteristic of most tumour cells is the resistance to agents that induce PCD in normal cells. This is often related to a variety of mutations in the genes regulating the PCD process. The inability to inactivate the bcl-2 gene upon a relevant stimulus produces cells reluctant to die. Wyllie et al has demonstrated that cells without the p53 gene are

resistant to cell death. Mice that lack the p53 gene often develop tumours. In cancer cells examined to date, the p53 gene is the one that is most often mutated (Schwartz 1993). Several tumour viruses such as adenovirus (E1b region), papillomavirus E6 and SV 40 inactivate the p53 gene. Since tumour cells are resistant to the normal PCD-inducing signals, there is a need for other substances that eliminate immature and potentially tumorigenic cells.

Human milk provides the breast-fed child not only with nutrients, but also with a mucosal immune system. Milk contains a wide array of molecules with anti-microbial activity; antibodies to bacterial, viral, and protozoal antigens (1-3), potentially bactericidal molecules like lysozyme and lactoferrin, fatty acids that lyse bacteria and viral particles and glycoconjugates that inhibit bacterial adherence to epithelial cells (4-6). These components reach mucosal surfaces in the respiratory and gastrointestinal tracts of the breast-fed child, and are thought to interfere with various steps in the pathogenesis of infections at these sites (5, 7). As a result, breast-feeding protects the infant from respiratory and gastrointestinal infections.

MATERIALS AND METHODS

Cellines

Epithelial cell lines from the human respiratory tract, gastrointestinal tract and urinary tract were used. The respiratory tract cell lines were a human lung carcinoma cell line, A549 (ATCC [American Type Culture Collection] CCL 185), and a human pulmonary mucoepidermoid carcinoma cell line NCI-H292 (ATCC CRL 1848). The urinary tract cell lines were A-498 (ATCC HTB 44), isolated from a kidney carcinoma, J 82 (ATCC HTB 1), from a transitional-cell carcinoma and 5637 (ATCC HTB 9), from a primary bladder carcinoma. The intestinal cell lines were CaCO-2 (ATCC HTB 37) and HT-29 (ATCC HTB 38), both isolated from a colonic epithelial-like adenocarcinoma. A dog kidney cell line derived from a normal cocker spaniel kidney MDCK (ATCC CCL 34) and two fibroblast-like cell line derived from normal African green monkey kidney Vero (ATCC CCL 81) and GMK were used for experiments involving TNF. The T1210 mouse leukaemia cell line was used for animal experiments.

The cell lines were cultured in 25 cm^2 cell culture flasks (Nunc,

Roskilde, Denmark) in RPMI 1640 (Gibco/BRL, Life Technology Ltd, Paisley Scotland, U.K) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 50 µg of gentamicin per ml. The cells were detached by trypsin treatment (0,25%; Sigma Chemicals Co., St. Louis, Mo.) for about 10 min at room temperature. Trypsinized cells were used to seed 96-well microtiter plates and were also maintained in suspension in maintenance medium (RPMI 1640 supplemented with 2% FCS, 2 mM glutamine and 50 µg of gentamicin per ml) and used for experiments as described below.

Non-transformed cells

Rat thymocytes were harvested as described (Zhivotovsky 20).

Human embryonal lung cells (Hel) and human foreskin fibroblasts (HFF) were obtained from the Section of Virology, Dept. of Microbiol., Lund University, Sweden. These cell types were cultured as described above for the cell lines.

Human nasopharyngeal epithelial cells were harvested from the upper respiratory tract of healthy donors by scraping the pharyngeal wall behind the soft palate with a cotton-tipped wooden swab. The cells were detached by gentle stirring in medium, washed twice by resuspension in medium and centrifugation at 500 x g.

Human urinary tract epithelial cells were collected from freshly, voided morning urine from a healthy donor. The cells were sedimented by centrifugation at 500 x g for 10 min, washed twice by resuspension in medium and centrifugation at 500 x g.

Cells from mouse kidney and bladder were obtained from homogenized tissues. The mice were sacrificed by cervical dislocation, the tissues were removed aseptically, placed in 5 ml of PBS in plastic bags and homogenized in a Stomacher Blender 80 (Seward Medical, London, UK). The cell suspensions were centrifuged at 500 x g for 10 min. Destilled water (5ml) was added to each tube for 30 sec. to lyse erythrocytes, after which time 5 ml of 0.30M NaCl was added to restore the osmolarity. The cells were harvested by centrifugation at 500 x g for 10 min and resuspended in medium.

Human peripheral blood lymphocytes and granulocytes, were isolated from one healthy donor by centrifugation of whole blood in a

Polymorphprep™ solution (Nycomed Pharma AS, Oslo, Norway) at 500 x g for 30min. The mononuclear and polynuclear cell fractions were diluted in one volume of 0.075M NaCl to restore osmolality, washed twice by centrifugation and resuspended in medium.

Human milk and human milk components

The purification of active material was performed from sterilised milk or alpha-lactalbumin, and all the purification steps were performed under sterile conditions.

Casein precipitation.

About 2L of heat-pasteurised milk were thawed at a time and centrifuged to remove fat. Casein was prepared by adding about 27ml of 10% potassium oxalate to each liter of fat free milk. After overnight incubation at 4°C, the precipitate was discarded. To the supernatant, 1mM HCl was slowly added to a final pH of 4.6. The solution was then heated to 30°C for 1h and subsequently left overnight at 4°C. The casein precipitate was separated by centrifugation and washed by 3-5 cycles of resuspension in distilled water and centrifugation. Finally the precipitate was lyophilized and resuspended to the concentration required for each experiment.

The pH of the whey fractions was readjusted with phosphate buffered saline (PBS) before testing for the cell killing activity.

Casein fractionation.

Proteins were fractionated on an ion-exchange column (cm X 1.5 cm) packed with DEAE-Trisacryl M (Pharmacia-LKB, Uppsala, Sweden) attached to a fast protein liquid chromatography (FPLC) instrument (Pharmacia). A NaCl gradient was used during the fractionation. The run was under the following conditions: buffer A: 0.01M Tris-HCl pH 8.5; buffer B: buffer A containing 1M NaCl/L. Gradient program: start 15% B; from 0-60 ml: 30% B; from 60-80 ml: 30% B for 10 min; at 80 ml: 100% B; from 80-90 ml: 100% B for 10 min; from 90-120 ml: 100% A. Flow rate: 1ml/min, recorder: 0.1cm/min. The buffers were degassed and filtered through 0.22mm filters before use. The peaks were monitored at 280nm, and the fraction size was 3ml. Fractions were pooled, desalted by dialysis (membrane cut off 3.5kD) against distilled water for at least 48h, lyophilized and tested for cell killing activity as follows.

The results are shown in Table 1. Fraction VI was tested in the *in vivo* studies referred to later.

Ion-exchange chromatography of commercial alpha-lactalbumin

20 mg of commercial (Sigma) human or bovine alpha-lactalbumin were dissolved in 2 ml 0.01 M Tris-HCl, pH 8.5. The ion-exchange chromatography of alpha-lactalbumin was carried out under similar conditions as described above for the fractionation of casein. The NaCl gradient was linear (not interrupted), flow rate was 1 ml/min, 3 ml fractions were collected and pooled as above. The pools were dialysed (membrane cut-off 3.5 kD), lyophilized, resuspended to the required concentration and tested for induction of cell death.

Analysis of the milk fraction

Polyacrylamide gradient gel electrophoresis (PAGGE). Analytical PAGGE was performed using 4-20% polyacrylamide precast gels (Bio-Rad, Richmond, CA) on a Bio-Rad's Mini Protean II cell. To 10ml (5-10mg/ml) each of the lyophilized fractions, an equal volume of sample buffer (13.1% 0.5M Tris-HCl, pH 6.8, 10.5% glycerol, 1.2% SDS and 0.05% Bromophenol Blue) was added. 20ml of each sample was then loaded on to the gel, which was run in Tris-glycine buffer (pH 8.3) with 0.1% SDS at 200V constant voltage for about 40min. Staining of the proteins was by immersing the gel in Coomassie Blue solution (0.1% in 40% methanol, 10% acetic acid) for about 0.5h. Destaining was by several changes in 40% methanol, 10% acetic acid until a clear background was obtained.

Electroblotting of proteins. After SDS-PAGGE; protein bands were transferred by Western blotting onto polyvinylidene difluoride (PVDF) membranes (Pro Blott Membranes, Applied Biosystems, CA), as described (13). The proteins were visualized by Coomassie Blue staining and the stained bands were cut out for protein sequencing.

Amino acid sequence determination. The PVDF-immobilized protein bands were subjected to Edman degradation in an automated pulse-liquid sequencer (Applied Biosystems model 477A).

Electrospray ionization mass spectrometry (ESI-MS) Purified proteins were analyzed on a VG Bio-Q ESI-MS (Fisons/VG, Manchester, UK) equipped with an atmospheric pressure electrospray ion source and a quadrupole

mass analyzer with a maximum mass range of 4000. The mass spectrometer was scanned from m/z 600 to m/z 2000 in 10secs. The mass resolution was set to 500. The data system was operated as a multichannel analyzer and 5 scans were averaged to obtain the final spectrum. The electrospray carrier solvent was 1% acetic acid in acetonitrile-water, 1:1, and the flow rate was 2-4µl/min. The sample was dissolved at a concentration of 10-20pmol/µl in the carrier solvent and 5µl was injected. The molecular weight of sample components was estimated from the m/z values of series of ions as described earlier (Mann et al., 1989).

Matrix assisted laser desorbtion ionization mass spectrometry (MALDI)

The purified protein was analyzed by MALDI mass spectrometry on a LDI 1700 time of flight mass spectrometer eqipped with a pulsed nitrogen laser (337 nm) (Biomolecular Separations Inc., Reno, Nevada). The laser power was set to 8.6µJ and the spectrum was th[illegible]n of 140 laser shots. Sinnapinic acid was used as a matrix and bovine serum albumin was used as the external standard. About 100µg of the protein fraction to be analyzed was dissolved in 50µl water and 0.1% trifluoroacetic acid (TFA). 10µl of this solution was mixed with 10µl 50mM sinnapinic acid. The probe was loaded with 0.8µl of the sample mixture, vacuum dried, loaded with another 0.8µl of sample, and vacuum dried again before being inserted into the mass spectrometer.

Morphology

Light microscopy

One drop of the cell suspension was placed on a microscope slide and allowed to air dry. The cells were fixed with methanol (99.5%) and stained using the May-GrŸnwald reagent. The morphology was examined in light microscopy at 800 x magnification.

Transmission electron microscopy

Cells exposed to medium or MAL were double fixed in glutaraldehyde plus osmium tetroxide, embedded in agar 100 and post-stained with uranylacetate and lead. The cells were sectioned and examined by transmission electron microscopy.

Viability testing

The viability of the cell lines was tested by thymidine incorporation.

The cell lines were grown to confluency in 96-well plates (Falcon, Becton-Dickinson, New Yersey, USA). At time zero, 100µl of medium was aspirated from each well, replaced by 100µl of the different experimental solutions and incubated at 37°C in an atmosphere of 5% CO_2 for 30min. Tritiated thymidine (0.5µCi, Amersham Life Science, Amersham, U.K) was added to each well and the cells were incubated for 4hours at 37°C in 5% CO_2. The medium and experimental solutions were discarded and the cells were washed twice in PBS. The cells were detached by trypsin treatment, harvested and the radioactivity was measured in a 1205 Betaplate™ Liquid scintillation counter (Wallac Oy, Finland).

The viability of the cell lines was also tested by trypan blue exclusion. The cells were harvested from the cell culture flasks by trypsin treatment as described above, washed twice by centrifugation at 500 x g and resuspension in medium; and the cell concentration was adjusted to 5 x 10^5 cells/ml. The cell suspension (50µl) and each of the experimental solutions (50µl) were mixed and incubated at 37°C in 5% CO_2 for 30min. One drop of a 0.2% trypan blue solution (Chroma-Gesellschaft Schmid & Co, Stuttgart, Germany) was added to each tube and the number of stained cells per 100 cells was determined by interference contrast microscopy (Orlolux II microscope with interference contrast equipment TE Leitz, Wetzlar, Germany).

The effect of human milk and milk components on the viability of non-transformed cells was analyzed by trypan blue exclusion. The cell concentration was adjusted to 10^5 cells/ml for nasopharyngeal and uroepithelial cells and 5 x 10^5 cells/ml for the remaining cell types by microscopic counting in a Bürker chamber. The cell suspensions (50µl) was mixed with each of the experimental solutions (50µl) and incubated at 37°C in 5% CO_2 for 30min. One drop of a 0.2% trypan blue solution was added to each tube and the number of stained cells per 100 cells was determined by interference contrast microscopy.

Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration.

A549 cells (4 x 10^7 cells) or rat thymocytes (5 x 10^7 cells) were incubated in Krebs-Henseleit buffer, pH 7.2, supplemented with 10 mM Hepes, 15 mM glucose, and 1% BSA. Prior to treatment, cells were loaded

with 5 µM fura-2AM for 25 min at 37°C, washed free of extracellular fura-2AM by centrifugation for 30 s at 1,500 x g, and resuspended in Krebs medium before measurement of $[Ca^{2+}]_i$ (Zhivotovsky 1993, Grynkiewicz). After a steady baseline was obtained, inducing agents were added to the cells. Control cells had only carrier solvent.

Influence of protein synthesis inhibition and Ca^{2+} in MAL-induced apoptosis.

To investigate the influence of protein synthesis inhibition viability testing and DNA fragmentation analysis was performed on cells pretreated for 30 min at 37°C with cycloheximide (5 µg/ml).

To investigate the importance of Ca^{2+} in the apoptotic pathway of MAL-induced apoptosis viability testing and DNA fragmentation analysis was performed in the absence of Ca^{2+} in the medium.

TNF-alpha bioassay

WEHI cells were grown to confluency in cell culture flasks, detached by Versene treatment (0.2g EDTA /l in PBS), washed in medium and diluted in medium to a final concentration of 4-8 x 10^5 cells/ml. The experimental solutions (50 µl) were added triplicate to a microtiter plate and mixed with 50µl of cell suspension. A standard curve was prepared using recombinant TNF-α (50µl/well, 400 ng/ml - 4pg/ml). After incubation for 20h at 37°C in 5% CO_2. 10 µl of MTT (5mg/ml; Sigma) was added to each well, the cells were incubated for 4h at 37°C, the supernatants were aspirated and 100 µl of propanol-HCl was added to lyse the cells. The samples were read at 570 nm in a Labsystems Multiskan spectrophotometer (source). A high absorbance indicates a high number of living cells. Neutralizing polyclonal anti-TNF antibody ( ) was kindly provided by I. Olsson, Dept. of Medicine, Lund University, Lund, Sweden.

DNA fragmentation.

Cells (2 x 10^6 cells) were lysed, centrifuged at 20,000 x g, and the supernatant was extracted with phenol-chloroform. Precipitated oligonucleosome length DNA fragments were loaded on 1.8% agarose gels, electrophoresed with constant current set at 60 mA, visualized with ethidium bromide using a 305 nm UV-light source and photographed using polaroid 665 positive-negative film (Zhivotovsky 1994a).

High molecular weight DNA fragments were detected by field-

Inversion gel electrophoresis (FIGE) as described (Zhivotovsky 1994a). Agarose gel electrophoresis was run at 180 V in 1% gels in 0.5 x TBE (45 mM Tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0), at 12°C, with the ramping rate changing from 0.8 s to 30 s for 24 h, using a forward to reverse ratio of 3:1. Gel staining and photography were as described above.

Experimental animals.

C57 BL/6J x DBA 2J F1 female mice, weighing 18-20 g, were purchased from Gl. Blomholdtgård, Ry, Denmark. The mice were given standard laboratory feed and tap water *ad libitum*.

Nude BALB/c (nu/nu) mice were bred by mating heterozygous (*nu/+*) females with homozygous (nu/nu) males. The mouse colony was kept under sterile conditions in sterile filter top cages with coarse sawdust bedding. They obtained sterilized standard laboratory feed and acidified water (pH 2.5-2.7) *ad libitum*.

Induction of L1210 tumors.

L1210 tumors were induced intraperitoneally or subcutaneously. L1210 cells were harvested from the tissue culture flasks by centrifugation at 500 x g for 10 min. Pelleted cells were washed once in 0.15 M NaCl and resuspended in 0.15 M NaCl. The cell concentration was adjusted by counting in a Bürker chamber.

Male and female BALB/c (*nu/nu*) mice and female C57BL/6J x DBA 2J F1 mice were inoculated *i.p.* with L1210 cells suspended in 0.6 ml of 0.15 M NaCl. The mice were inspected every 12h for the development of ascites and the survival was followed. Surviving mice were sacrificed after 28 days.

The BALB/c (*nu/nu*) mice were injected *s.c.* on each flank with L1210 cells in 0.3 ml of 0.15 M NaCl per injection site. The mice were inspected every 3 days and the day of reaching 25 x 25 mm tumors was determined. The mice were sacrificed when the tumor size reached 25 x 25 mm or after a total of 28 days. The protocols were approved by the animal ethics comittee, Lund, Sweden.

Prevention of tumor development by treatment with MAL

a) **In vitro pretreatment of L1210 cells with MAL**

L1210 cells were preincubated with MAL suspended in sterile RPMI

medium for 3h *in vitro*. The cells were washed by centrifugation at 500 x g, resuspended in 0.15 M NaCl at 1.67 x 10^8 cells per ml and 0.6 ml (1 x 10^8 cells) were injected *i.p* in 10 animals. Control animals (n = 10) received L1210 cells preincubated without MAL. MAL pretreated cells (5 x 10^5)were also injected *s.c* on each flank in a group of 10 animals. Control animals (n = 10) received L1210 cells pretreated with RPMI.

b) **Sequential injection of L1210 cells and MAL.**

L1210 cells (1 x 10^5) were suspended in 0.3 ml 0.15 M NaCl and injected into the peritoneal cavity in two groups of ten animals each. MAL was dissolved in NaCl and 0.3 ml of MAL solution was injected at time 0 or 24 h after injection of tumor cells. Control animals received NaCl alone. L1210 cells (5 x 10^5) were suspended in 0.3 ml 0.15 M NaCl and injected subcutaneous on each flank in a group of 10 animals. 0.3 ml of MAL solution was injected at time 0 or 24 h after injection of tumor cells. Control animals received NaCl alone. In each case in these *in vivo* studies the MAL was fraction IV of Table 1 calculated as its solids content.

Statistics.

The Mann-Whitney U test was used for unpaired, nonparametric column comparisons.

RESULTS

Human milk and human milk fractions induce cell death in four human cell lines.

The ability of human milk and human milk fractions to kill cells was first observed using the A549 cell line. Cells exposed to human milk were small and had a condensed nucleus as compared to the control cells. Human milk reduced the growth of the A549 cells by 99.6% as determined by thymidine incorporation and the viability by 97.6% as determined by trypan blue exclusion (Table 1). Bovine milk had som growth inhibitory effect (43%) but did not affect cell viability (Table 1).

Human milk was fractionated in order to localize the cytotoxic component(s). The cytotoxic activity precipitated with the casein; none remained in the whey fraction (Table 1). At a concentration of about 5

mg/ml, casein reduced the viability of the A549 and MDCK cells from 95% to 0% in 30 min.

Casein was further fractionated by ion-exchange chromatography. 100 mg of the lyophilized casein was dissolved in 10 ml 0.01 M Tris-HCl, pH 8.5, centrifuged and applied directly on the ion-exchange column. Six fractions were obtained (I-VI) which were pooled, desalted, lyophilized and reconstituted to a concentration of about 5-10 mg/ml. The cell killing activity was concentrated in pool VI which eluted after 1 M NaCl. This pool reduced the viability of A549 cells by approximately 100% in 30min. (Table 1)

Characterization of the active fraction (fraction VI)

Pool VI was analyzed by PAGGE which revealed the presence of bands with a molecular mass of 14 kDa, 28 kDa and 100 kDa. Proteins after PAGGE were transferred to a polyvinylidene difluoride membrane by Western blotting, and identified by N-terminal amino acid sequencing. The higher molecular mass bands of fraction VI and the 14 kDa band showed complete N-terminal sequence homology with the known sequence of human α-lactalbumin and with the sequence of a commercial sample of α-lactalbumin (data not shown). Examination of the fraction by ESI-mass spectrometry revealed that the estimated molecular mass of the major component of the active protein fraction (14.088 kDa) and of monomeric α-lactalbumin (14.081 kDa) were both close to the molecular mass of α-lactalbumin calculated from the amino acid sequence (14.078 kDa). The small differences rule out most known posttranslational modifications and suggest that the major component of this fraction did not differ in its covalent structure from α-lactalbumin. MALDI mass spectometry showed a major peak close to 14 kDa consistent with monomeric α-lactalbumin, but also peaks at 28 kDa and 42 kDa which is consistent with the di- and trimeric forms.

Monomeric, commercial human or bovine α-lactalbumin had no effect on cell viability (Table 1). The active fraction was also incubated with polyclonal anti-α-lactalbumin antibodies, but no inhibitory effect could be seen as compared to the active fraction. However, when monomeric α-lactalbumin was converted to the multimeric form by passage over the ion-exchange column under conditions used to fractionate casein, the

multimerized α-lactalbumin became cytotoxic in the cell assays (Table 2) Other human milk proteins (lactoferrin and lysozyme) were found to be inactive at a concentration of 10 mg/ml (Table 1). Thus, we conclude that a multimeric complex of a-lactalbumin (MAL) in human milk induces cell death.

Apoptosis in relation to cell type

Cell lines of human, monkey, murine and canine origin were incubated with multimeric α-lactalbumin (final concentration 5mg/ml) monomeric α-lactalbumin (final concentration 5mg/ml) or with medium. The viability was examined by thymidine incorporation (Table 3). A decrease in DNA synthesis occurred in all the cell lines tested. The viability was also examined by trypan blue exclusion. A decrease in viability by at least 99% as determined by thymidine incorporation and 85% as determined by trypan blue exclusion occured in all cell lines tested. There was no effect on cell viability when exposing the cells to monomeric a-lactalbumin (Table 3).

The effect of multimeric α-lactalbumin on cell viability was subsequently analyzed using cells from different mammalian tissues. The cells were incubated with multimeric α-lactalbumin, monomeric α-lactalbumin or medium and the viability was examined by trypan blue exclusion. Embryonic human cells (Hel, HFF) and human peripheral blood lymphocytes were sensitive to treatment with multimeric α-lactalbumin; their viability decreased to 8%, 8% and 16%, respectively (Table 3). Peripheral blood granulocytes showed intermediate sensitivity to multimeric α-lactalbumin and retrieved 62% viability (Table 3). In contrast, human epithelial cells from the upper respiratory tract and the urinary tract, as well as mouse kidney and bladder cells were resistant to the effect of multimeric α-lactalbumin and retained full viability after incubation with this protein (Table 2). Monomeric α-lactalbumin had no effect on cell viability for any of the non-transformed cells tested.

MAL induces apoptosis

Oligonucleosome-length DNA fragments characteristic of apoptosis were observed in thymocytes treated with 0.3, 0.5, and 0.7 mg/ml of MAL for 4 h, and in MDCK cells treated with 1 mg/ml of MAL for 24 h. Fragmentation increased with the concentration of MAL. In A549 cells MAL

produced HMW DNA fragments of the kind that is seen early in thymocyte apoptosis (17). DNA ladders were not observed in A549 cells even after higher concentrations of MAL (5 mg/ml) or prolongation of the incubation time (48 h) (data not shown).

Mechanisms of MAL induced apoptosis

TNF and complement. Monomeric α-lactalbumin is structurally related to lysozyme and lactoferrin, two proteins present in human milk. Human lysozyme, chicken lysozyme, or bovine lactoferrin were tested and found not to have any effect on cell viability (Table 4).

Milk has been reported to contain TNF as defined by death of the WEHI cell line (ref). WEHI cells were exposed to multimeric α-lactalbumin, human milk, and bovine milk. TNF was used as a control (400 ng/ml). The effect of TNF on the WEHI cell viability was reversed by anti-TNF antibodies. Multimeric α-lactalbumin and human milk killed the WEHI cells; this effect was not revesed by anti-TNF antibodies (Table 4). MDCK cells were killed by multimeric α-lactalbumin but not by TNF.

Human milk has been reported to contain complement components. The effect of MAL was not decreased by heating to 56°C for 30 min (Table 4), suggesting that the effect was unrelated to complement.

Protein synthesis inhibition. In many experimental systems the induction of apoptosis is accompanied by *de novo* mRNA and protein sythesis and is blocked by protein synthesis inhibitors (Wyllie, McConkey). Cycloheximide effectively prevented MAL-induced DNA laddering in MDCK cells (data not shown) and cell death in A549 and MDCK cells. Thus, in the presence of cycloheximide 82% of A549 cells and 79% of MDCK cells excluded trypan blue, as compared to 22% of A549 cells and 8% of MDCK cells exposed to MAL in the absence of cycloheximide.

Calcium fluxes. In an attempt to characterize the biochemical events involved in milk protein-induced cell death, we studied the effect of MAL on $[Ca^{2+}]_i$ in both A549 cells and rat thymocytes; $[Ca^{2+}]_i$ was assessed using fura-2 (Grynkiewicz), and with the SERCA Ca^{2+}-ATPase inhibitor, thapsigargin, as a positive control (Thastrup, Jiang). This compound has been found to cause a sustained $[Ca^{2+}]_i$ increase and to promote all the characteristic features of apoptosis in rat and human thymocytes (Jiang,

Zhivotovsky 1994b). Thapsigargin rapidly induced a sustained increase in $[Ca^{2+}]_i$ in A549 cells. Thapsigargin (100 nM) also produced DNA fragmentation in both A549 cells and rat thymocytes after incubation for 24 h and 4 h, respectively.

We found that MAL induced a concentration dependent increase in $[Ca^{2+}]_i$ after 30 s exposure of A549 cells. This increase was transient and dropped considerably after 2 min of exposure. MAL induced a concentration dependent increase in $[Ca^{2+}]_i$ also in rat thymocytes. This increase reached a maximum after 30 s of exposure and was maintained at this level of $[Ca^{2+}]_i$ during the next 5-6 min. In order to evaluate the importance of the observed increase in $[Ca^{2+}]_i$ for the cytotoxic activity of MAL, the experiments were repeated under calcium-free conditions. Cells (2×10^5) were incubated with 1 mg/ml of MAL with or without Ca^{2+} in the medium, the viability was tested and the cells were subjected to DNA purification and gel electrophoresis (data not shown). Cell death was prevented under calcium-free conditions. The viability of untreated MDCK cells during incubation in complete or Ca^{2+}-free medium was 98%. Treatment with MAL in complete medium decreased the viability to 75%, whereas MAL treatment of MDCK cells in Ca^{2+}-free medium caused only a minor decrease in cell viability (to 92%). These results were confirmed by measurement of chromatin cleavage using FIGE. Formation of HMW DNA fragments induced by MAL in MDCK cells was prevented by removal of Ca^{2+} from the medium (data not shown).

Protection against tumor development in BALB/c (*nu/nu*) mice.

A) **In vitro pretreatment of L1210 cells with MAL**

For intraperitoneal injection, L1210 cells were preincubated with sterile MAL (0.5 mg/ml) for 3h. The cell suspension was washed and resuspended in NaCl and 1×10^6 cells were injected *i.p* into ten mice. Mice injected with untreated cells suspended in NaCl served as a control. The difference in survival is shown in Fig 1. The control mice had a median survival of 12 days (range 9-21 days). Survival in mice receiving the MAL treated cells was increased by 9 days (median survival 21 days, range 17-28, $p < 0.001$) and three mice survived the entire test period.

For subcutaneous injection, L1210 cells were preincubated with MAL

as described, washed, resuspended in NaCl and injected *s.c.* Mice receiving untreated cells served as controls. The difference in tumor development is shown in Table 5. The controls developed 25 x 25 mm tumors by day 13 (range 9-15 days). None of the animals receiving MAL treated cells developed tumors.

B) **Sequential administration of L1210 cells and MAL.**

Nude mice were injected *i.p* with 1 x 10^6 L1210 cells followed by a sterile aqueous solution of MAL (4 or 8 mg MAL per mouse). Mice injected with cells suspended in NaCl served as controls. Controls receiving MAL but no cells were included. MAL treatment was shown to drastically enhance the survival of the mice. While the control mice had a median survival of 10 days (range 9-16 days), animals treated with 4 and 8 mg of MAL had an increased life span (ILS) of 9 and 6 days respectively (Fig 2). Mice receiving MAL but no cells survived the experimental period.

Protection against tumor development in syngeneic mice.

A) **In vitro pretreatment of L1210 cells with MAL.**

C56 BL/6J x DBA 2J F_1 mice were injected intraperitoneally with 1 x 10^6 L1210 cells, pretreated with sterile MAL or NaCl as described. The control mice developed ascites and had a median survival of 11 days (range 8-11 days) All except one of the mice receiving MAL treated cells survived till the end of the experimental period (Fig 3). The sick mouse died on day 19.

B) **Sequential administration of L1210 cells and MAL.**

Syngeneic mice were injected *i.p* with 1 x 10^6 L1210 cells followed by a sterile aqueous solution of MAL (8 mg MAL per mouse). Mice injected with untreated cells suspended in NaCl served as controls. Controls receiving MAL but no cells were included. MAL itself did not affect the survival of the mice. MAL treatment was shown to drastically enhance the survival of the mice. While the control mice had a median survival of 11 days (range 9-12 days), animals treated with 8 mg of MAL had an ILS of 17 days (Fig 4). Two mice receiving MAL treatment died during the experimental period whereas the remaining mice survived the entire experimental period.

DISCUSSION

Human milk was shown to induce apoptotic changes in transformed epithelial cell lines, but not in non-transformed epithelial cells of human origin. The loss of viability was defined by complete inhibition of thymidine uptake, and by the uptake of trypan blue. Apoptosis was accompanied by changes in cellular morphology, including apoptotic bodies and cell shrinkage and by DNA fragmentation. The apoptosis inducing component was identified as a multimeric form of human α-lactalbumin (MAL).

The purification of MAL from human milk by anion-exchange chromatography was monitored by the biological activity of each fraction. SDS-PAGGE profiles of the cytotoxic fraction showed a major band at 14 kDa and minor bands in the 30 kDa and 100 kDa regions. The N-terminal sequence of each band was identical to the sequence of α-lactalbumin, but α-lactalbumin itself lacked cytotoxic activity. However, the presence of additional minor bands on SDS-PAGGE suggested that the active component may exist in a different aggregation state. By ESI-MS analysis, the molecular weights of the active component (14.068) and of α-lactalbumin (14.081) were found to be similar, and the small differences ruled out most known posttranslational modifications. Analysis of the active protein by MALDI-MS showed peaks consistent with mono-(14 kDa), di-(28 kDa) and trimeric (42 kDa) forms of α-lactalbumin, suggesting that the apoptosis-inducing component was possibly a multimeric form of human α-lactalbumin.

MAL, was shown to protect mice against the development of L1210 leukemias. Tumors were induced in nude BALB/c mice as well as syngeneic C57BL/6J x DBA 2J mice, by subcutaneous or intraperitoneal injection of L1210 cells. Tumor development was scored by survival of the mice or by measurement of tumor size. The L1210 cells were sensitive to MAL, and underwent apoptotic cell death after exposure to MAL *in vitro*. Protection was induced by treatment of the L1210 cells with MAL *in vitro* prior to injection into the mice, but also by injection of MAL subsequent to tumor induction.

The L1210 lymphoma model in mice is well established. The tumor was first described in 1949 (10) and was initially maintained by passage in

mice. Methods for *in vitro* suspension culture were reported about 20 years later (11, 12). The intraperitoneal model for deliberate induction of tumors has been extensively used for routine screening of potentially cytotoxic agents. During the first 6 to 12 h after intraperitoneal injection about half of the cells migrate from the peritoneal cavity to other organs where they proliferate (13). The remaining cells form microtumors in the peritoneal cavity and ascites fluid is produced. The ability of L1210 cells to also induce subcutaneous, solid tumors was observed in this study. The kinetics of solid tumor development were similar to the intraperitoneal tumor, but the mice survived longer.

The protective effect of MAL was first observed after administration of cells that had been pretreated with MAL *in vitro*. The *in vitro/in vivo* approach was chosen to certify that MAL had interacted with the L1210 cells and that protection resulted from a direct effect of MAL on the cells rather than from activation of anti-tumor host defenses by injection of MAL.. The 3h pretreatment was used as earlier experiments had shown that 3h was the critical time to induce. The difference in survival between the mice receiving control cells and those receiving pretreated cells could not be entirely explained by the difference in cell viability of the inocula. Pretreatment with MAL reduced cell viability by about 50%, but it is likely that apoptosis had been triggered also in cells that remained viable, since continued in vitro incubation resulted in complete killing after six hours. According to Hofer and Hofer the doubling time of L1210 cells in C3H x DBA/2J F1 mice increased from an initial 8.5-9.5 h during the first 3 days to 34 h from day 5 and onwards after an inoculum of 1×10^6 cells (13). The difference in survival time therefore implies that the mice receiving pretreated cells died from an inoculum corresponding to 4000 cells. Thus the mice did not appear to possess mechanisms that could rescue cells that had been triggered by MAL to undergo apoptosis, even if these cells remained partially viable at the time of injection.

Some interesting discrepancies were observed between the *nu/nu* and syngeneic mouse backgounds. While there was no difference in the time to tumor development, there was a difference in survival. The syngeneic mice that received MAL pretreated L1210 cells i.p survived

beyond the end of the experimental period while *nu/nu* mice died. This implied that the syngeneic mice were able to kill the few tumor cells that survived after preincubation with MAL. The mechanisms of killing need to be identified.

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TABLE 1 - The influence of human milk and human milk components on the viability of four human epithelial cell lines.

	Cell viability						
	A549		NCI		A-498		
	cpm†	%¶	cpm†	%¶	cpm†	%¶	c
Medium control	21750	92	28500	91	45400	90	
Human milk							
Donor 1	75	2	23	1	35	3	
Donor 2	120	n.d	15	n.d	105	n.d	
Donor 3	400	n.d	15	n.d	420	n.d	
Fractions of human milk, donor 1							
Whey, donor 1	9500	92	32000	n.d	44400	n.d	
Casein, donor 1	100	0	110	0	60	0	
I	20200	n.d	29300	n.d	50500	n.d	
II	19700	n.d	16200	n.d	13500	n.d	
III	15000	n.d	20300	n.d	52100	n.d	
IV	24200	n.d	17200	n.d	31100	n.d	
V	15800	n.d	18600	n.d	53900	n.d	
VI*	50	0	15	0	40	1	
Organic phase		0		0		1	
Controls							
Bovine milk	12400	100	11300	97	11400	96	
α-lactalbumin, human	17375	92	30300	88	31500	84	1
α-lactalbumin, bovine	17600	93	24700	87	37200	86	1
lysozyme, human	14900	87	30800	91	29800	82	
lysozyme, chicken	16300	86	17500	86	43250	81	1
lactoferrin, bovine	n.d	98	32	n.d	908	n.d	
Multimerized α-lactalbumin	n.d	0					

* - Multimeric α-lactalbumin

† - Determined by thymidine incorporation. Mean of two separate experiments.

¶ - Determined by trypan blue exclusion. Mean of two separate experiments

TABLE 2. Influence of viability of A549 cells exposed to α-lactalbumin and activated fractions of α-lactalbumin after anion-exchange chromatography.

	Cell viability, %*	
	T.b.e†	T.‡
Medium control	92	100 (21750)
MAL	0	0
α-lactalbumin, human	92	80
Anion-exchange fractions		
α-lactalbumin, fraction 1	93	82
α-lactalbumin, fraction 2*	0	0

* containing multimerized α-lactalbumin

† Viability determined by thymidine incorporation

‡Viability determined by trypan blue exclusion

TABLE 3. Effects of MAL on the viability of different cell types.

Cells	Cell viability* Medium %†	Medium cpm‡ (100%)	MAL %†	MAL cpm‡ (%)
Cell lines				
A549, human lung	95	21750	0	50 (0)
NCI, human lung	91	28500	0	15 (0)
A-498, human kidney	84	45400	5	40 (0)
J 82, human bladder	92	7800	4	20 (0)
Caco-2, human intestine	98	18700	0	40 (0)
HT-29, human intestine	99	48400	6	75 (0)
HTB9, human kidney	98	11200	4	40 (0)
GMK, monkey kidney	72	14500	0	50 (0)
Vero, monkey kidney	83	45400	12	400 (1)
MDCK, dog kidney	95	53700	4	15 (0)
B9, mouse	100	35400	0	20 (0)
WEHI, mouse	90	14400	1	20 (0)
Embryonic cells				
Hel, human lung	67	-	8	-
HFF, human foreskin	90	-	8	-
Non-transformed cells		-		
Nph epithelium, human	43	-	45	-
Urinary epithelium, human	77	-	75	-
Granulocytes, human	100	-	62	-
Lymphocytes, human	100	-	26	-
Kidney, mouse	46	-	62	-
Bladder, mouse	38	-	48	-
Thymocytes, rat	100	-	0	-

* Cell viability was monitored byt thymidine incorporation and trypan blue exclusion. The viability of the embryonic and non-transformed cells was assayed only by trypen blue exclusion, since these cell types did not incorporate thymidine.
† % viability as determined by trypan blue exclusion.
‡ cpm as determined by thymidine incorporation. The medium control denotes 100%, numbers in parenthesis denotes as cpm sample x 100/cpmcontrol. Mean of three separate experiments.

TABLE 4 - Effect of complement and TNF on viability of A549 and WEHI cells.

	Viability (%)‡		TNF bioassay (abs)	
			Control	+ anti-TNF antibodies
	MDCK	WEHI	WEHI	WEHI
Medium	98	96	1.005	1.025
TNF (400ng/ml)	94	10	0.277	0.975
Multimeric α-lactalbumin				
5mg/ml	0	0	0.091	0.105
3mg/ml	0	0	0.096	0.106
2mg/ml	0	0	0.085	0.097
1mg/ml	35	12	0.355	0.322
5mg/ml, 56°C for 30min	3*	1*	0.160	n.d†

‡ Viability tested by trypan blue exclusion after 20h incubation.

† not determined.

* Viability tested by trypan blue exclusion after 30min incubation.

TABLE 5. Tumor development of subcutaneous injection of L1210 cells in nude mice

Treatment*	No. of mice**	Day of reaching 25 mm tumor growth; median (range)
Control	8	13 (9-15)
MAL	9	- †

* L1210 cells were preincubated for 3h with or without MAL before injection.

** Two control mice and one MAL-treated mouse died early in the experiment.

† No MAL-treated mice developed tumors.

CLAIMS.

1. A sterile aqueous solution of a multimer of α-lactalbumin.
2. A sterile aqueous solution of a multimer of α-lactalbumin suitable for injection into human beings for cancer therapy.
3. A solution according to claim 2 which comprises of 0.1 to 200 g/litre of multimers of α-lactalbumin.
4. A solution according to claim 3 which also comprises monomeric α-lactalbumin.
5. A sterile injectable composition for use in the treatment of cancer in mammals which comprises multimeric α-lactalbumin in a pharmaceutically acceptable diluent.
6. A composition according to claim 5 in which the pH is 6 to 8.
7. A method of processing human body fluids outside the human body which comprises adding a sufficient quantity of a multimer of α-lactalbumin to kill substantially alla of any cancer cells contained in the body fluid.
8. A composition which comprises a human body fluid outside the human body and a sufficient quantity of a multimer of α-lactalbumin to kill substantially all of any cancer cells contained in the body fluid.
9. The use of a multimeric α-lactalbumin in the preparation of a sterile injectable composition for use in cancer therapy.
10. A composition according to claim 5 which is contained in a sealed container.
11. A composition in a sealed container as claimed in claim 10 in which the container is a preloaded syringe or an intravenous infusion bag.
12. A composition in a sealed container according to claim 5 which contains 0.15 to 15 g of multimeric α-lactalbumin.
13. A sterile composition which composes a solid containing multimeric α-lactalbumin for insertion into a mammalian body which acts as a controlled release source of multimeric α-lactalbumin.
14. A method of treatment of a mammal suffering from cancer which comprises administering to it an effective amount of a multimeric α-lactalbumin.
15. A method according to claim 14 in which the mammal is a primate.

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16. A method according to claim 15 in which the mammal is a human.

17. A method according to claim 16 in which the multimeric α-lactalbumin is injected into the human body as a sterile aqueous solution thereof.

18. A method according to claim 16 in which the multimeric α-lactalbumin is contained in a controlled release composition which is implanted into the body.

19. A composition according to claim 5 which is a fraction mamalian milk in which the α-lactalbumin is multimerised producing an anti-cancer therapeutically effective composition.

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FIG 1 OF 4

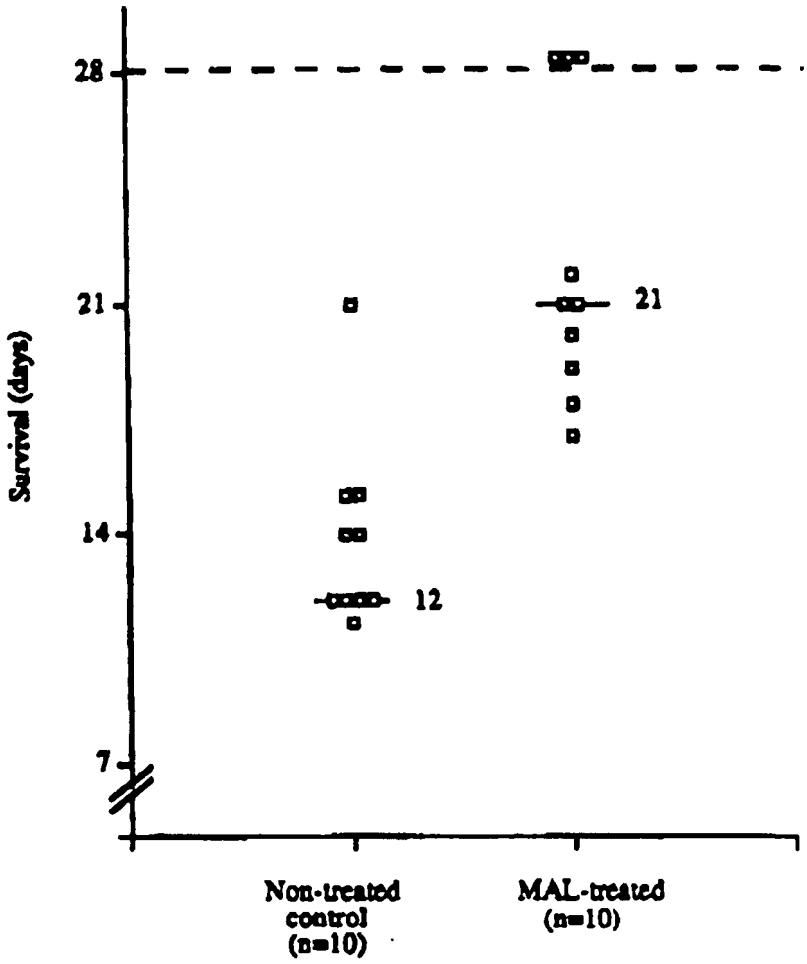

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FIG 2 OF 4

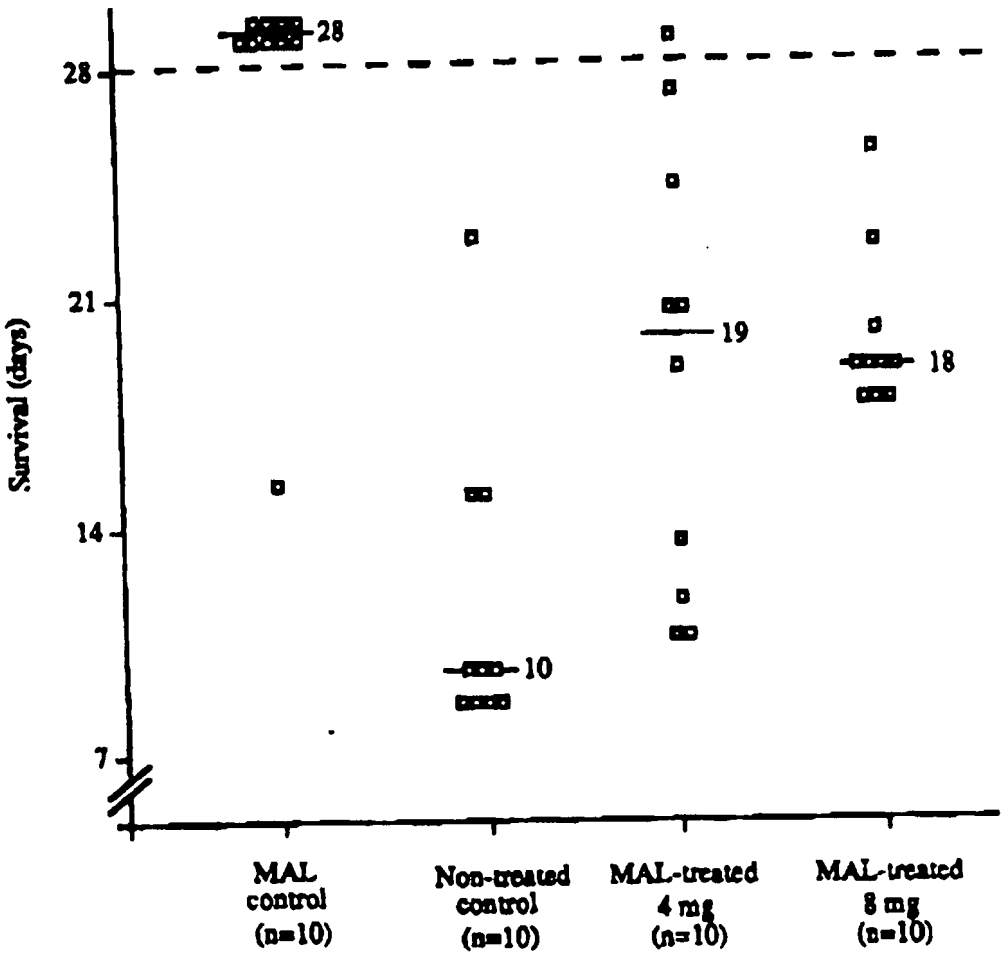

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FIG 3 OF 4

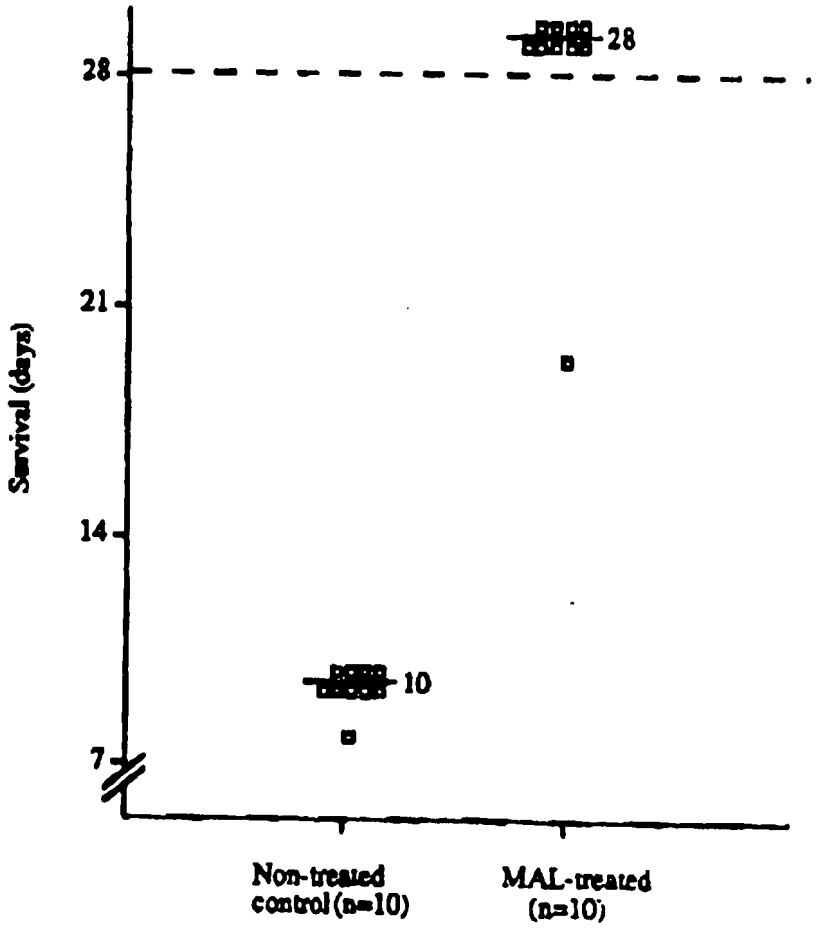

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FIG 4 OF 4